Mechanical stimulation regulates differentiation of bone marrow stem cells by modulating miR-140-5p via TGFβ1/Smad2 signaling pathway

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Research article

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Abstract

Background: Osteoporosis is a common progressive bone disease that drastically impairs patient health, independent mobility, and quality of life, and there is an urgent need for improved preventive and therapeutic strategies. A shift in bone marrow mesenchymal stem cell (BMSC) differentiation from osteogenic to adipogenic may contribute to disease pathogenesis. Mechanical stress on BMSCs is reported to promote osteogenesis, so we examined the effects of mechanical stimulation on BMSC differentiation and associated signaling pathways.

Methods: A sinusoidal tensile stress loading device was developed and examined effects of mechanical (stretch) stimulation on cultured BMSC (isolated from Sprague-Dawley rats) phenotype under osteogenic and adipogenic culture conditions. Osteogenic differentiation of BMSCs was assessed by alkaline phosphatase (ALP) staining and expression of Runx2 and BMP2, while adipogenic differentiation was evaluated by oil red O staining and expression of PPARγ and C/EBPα.

Results: It demonstrated that appropriate mechanical stimulation could promote osteogenic differentiation of BMSCs and inhibit differentiation into adipocytes. The mechanic stimuli could inhibit the expression of miR-140-5P in BMSCs, and the overexpression of miR-140-5P inhibited the osteogenic differentiation, whereas the inhibition of miR-140-5P promoted the osteogenic differentiation. Further, both mechanical stimulation and miR-140-5p knockdown promoted TGFβ1/Smad2 signaling, while miR-140-5p overexpression downregulated TGFβ1/ Smad2 signaling.

Conclusions: Appropriate mechanical stimulation promoted osteogenic differentiation and inhibited the adipogenic differentiation of BMSCs by lowering miR-140-5p expression, which in turn upregulates the TGFβ1/Smad2 signaling pathway. Our results provide a foundation for the development of effective strategies to promote bone remodeling, thereby lowering the burden of osteoporosis.

Background

Osteoporosis is a common progressive disease characterized by bone fragility due to the degradation of bone microstructure and decreased bone mass [1], which severely impacts health, mobility, and general quality of life [2]. Although the precise pathogenesis remains unclear, the fundamental mechanism underlying osteoporosis is an imbalance between bone formation and resorption [3]. Osteoblasts derived from bone marrow mesenchymal stem cells (BMSCs) serve to replenish bone tissue. However, BMSCs can differentiate into a variety of cell types aside from osteoblasts, including chondrocytes, myocytes, and adipocytes [4, 5]. In general, the shift in the differentiation from the osteogenic to adipogenic lineage increases with age, which may impair bone remodeling and reduce osteoblast formation, leading to the development of osteoporosis [6, 7].

Accordingly, most current drug treatments for osteoporosis inhibit bone resorption, accelerate bone formation, or promote bone mineralization. However, long-term use of these drugs has certain limitations. For instance, hormone replacement therapy may increase the risk of cardiovascular diseases [8], while

bisphosphonate therapy may cause severe bone turnover suppression and mandible osteonecrosis [9]. Thus, there is a critical need for improved preventive and therapeutic strategies for osteoporosis.

Most tissues are subjected to mechanical stimuli that regulate biological functions such as proliferation and differentiation [10]. Previous studies have shown that lack of exercise and the associated reduction in stem cell mechanical stimulation decreases osteogenic differentiation and increases the risk of osteoporosis [11, 12]. Indeed, our previous studies have demonstrated that mechanical stress can promote osteogenic differentiation and inhibit adipogenic differentiation of BMSCs [13, 14]. Further understanding of how mechanical stimuli, especially mechanical stress, affect skeletal tissue differentiation will provide insight into bone repair processes that may be exploited for novel therapeutic strategies. Several studies examining the relationships between mechanical stimulation and molecular expression profiles during bone healing have reported that non-coding microRNAs (miRNAs) participate in the regulation of skeletal growth and development. For instance, miR-140-5p has been reported to regulate cartilage development and bone homeostasis as well as contribute to age-related joint disease [15]. MicroRNA-140-5p was also found to be upregulated in murine primary osteoblasts and a vitamin D-treated osteoblast cell line [16]. A very recent study provided evidence that miR-140-5p regulates temporomandibular joint osteoarthritis (TMJ-OA) pathogenesis through the TGF-β/Smad2 signaling pathway [17]. Furthermore, miR-140-5p was reported to promote osteogenesis of ACSs by directly regulating toll-like receptor 4 (TLR4) and bone morphogenic protein 2 (BMP2), resulting in enhanced fracture healing and bone formation in the atrophic nonunion rat model [18]. However, is still unknown whether miR-140-5p influences osteogenic or adipogenic differentiation of BMSCs in response to mechanical stimulation. The purpose of this study is to investigate the effects of mechanical stimulation on osteogenic and adipogenic differentiation of BMSCs and to explore the underlying molecular mechanisms, including the contributions of miR-140-5p and TGF-β/Smad2 signaling.

Methods

Isolation and culture of rat BMSCs

Bone marrow stem cells were isolated from Sprague-Dawley rats (Experimental animal center of Nanfang Hospital，male or female, 80–100g. All rats were killed by cervical dissection, and the bodies were collected and burned by environmental health management department of Guangdong province.) by flushing the femurs and tibias with DMEM-LG medium (Gibco, Langley, OK, USA) supplemented with 10% defined fetal calf serum (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin (North China Pharmaceutical Factory, China) (termed general medium, GM). Isolated cells were plated in the some medium on 25 cm^2 flasks and incubated at 37 °C under a humidified atmosphere containing 5% CO_2. After 24 h, non-adherent cells were removed by washing with PBS, and fresh GM was added to allow for further growth. The culture medium was changed every 2–3 days thereafter. When the cells reached 80%–90% confluence, they were washed with PBS, detached by 0.25% trypsin, and subcultured in new 25 cm^2 flasks at 1×10^4 cells/cm^2. Cells were collected at the second or third generation and sent for flow

cytometry analysis (BD, Franklin Lakes, NJ, USA) of CD29, CD34, CD44, and CD45 expression for confirmation of BMSC phenotype [13, 14].

Induction of osteogenesis or adipogenic differentiation and delivery of mechanical stimuli

Bone marrow stem cells at the logarithmic growth phase were collected, washed, and resuspended in DMEM at 1×10^5 cells/ml. Cells were seeded onto Bioflex 6-well plates at 1.5–2 ml per well and incubated under 5% CO_2 at 37 °C. Control cultures were refreshed with basal medium every 48 h. Differentiation of BMSCs was induced using the culture conditions previously described [19]. For osteogenic induction, cells were cultured in medium supplemented with 10% FBS, 1% penicillin/streptomycin, 100 nmol/L dexamethasone, 50 μmol/L ascorbic acid 2-phosphate, and 10 mmol/L β-glycerophosphate. For adipogenic induction, cells were cultured in DMEM containing 10% FBS, 1% penicillin/streptomycin, 1 μmol/L dexamethasone, 0.5 mmol/L IBMX, 10 mg/L insulin, and 200 μmol/L indomethacin. Mechanical stimulation parameters were set to 5% deformation rate, 0.5 Hz, and 6 h/d. The mechanical stretching device was updated to stimulate cells as previously described [20].

Alkaline phosphatase and oil red O staining

An ALP staining kit (Tiangen Biotech Co. Ltd., Beijing, China) was used for determination of osteogenic differentiation according to the manufacturer´s instructions. Briefly, cells were fixed with 4% paraformaldehyde in PBS for 12 min at room temperature then stained with 1–2 ml/well ALP solution for 30 min at room temperature. The solution was aspirated and the cells were washed with distilled water and observed under light microscopy for ALP-positive cells. For determination of adipogenic differentiation, cells were fixed with 10% neutral buffered formalin for 1 h at room temperature, incubated with 60% isopropanol for 1 min, and then stained with oil red O for 15 min. Positively stained lipid droplets (red) were visualized under light microscopy.

Western Blotting

Cells were lysed by incubation in RIPA buffer containing protease inhibitor cocktail (Roche, Shanghai, China) on ice for 30 min. Cell lysates were centrifuged at 12000 g for 5 min at 4°C, and the supernatant was collected. Protein concentration was determined using the Pierce BCA protein Assay Kit (Thermo Scientific, MD, USA). Total protein was separated by SDS-PAGE (25 μg per gel lane) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, MA, USA). Membranes were blocked with 5% non-fat milk in TBST buffer and probed with primary antibodies against the osteogenic markers Runx2 (Biorbyt, CA, USA) and BMP2 (ab14933, Abcam) or the bone adipogenic markers PPARγ (ab45036, Abcam) and C/EBPα (ab40764, Abcam). In addition, membranes were incubated with anti-Smad2 (ab33875, Abcam), anti-TGFβ1 (ab179695, Abcam), and anti-TGFR1 (ab31013, Abcam) to assess expression of TGFβ1/Smad2 signaling pathway components. Subsequently, membranes were washed with TBST, and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (115-035-003 and 111-035-003 from Jackson Immuno Research Laboratories, West Grove, PA, USA). Target

proteins were visualized using a ECL chemiluminescence kit (Pierce) and band densities quantified using ImageJ (National Institutes of Health, USA).

RNA Extraction and RT-PCR Analysis

The expression levels of osteoblastic and adipocyte genes were analyzed by quantitative real-time PCR. Briefly, BMSCs were harvested and RNA was extracted using Trizol Reagent. Total RNA was reverse transcribed to cDNA using kit K1622 (Thermo Scientific). Real-time PCR assays were performed using All-in-One™ qPCR reagents (Genecopoeia, Guangzhou, China) with specific primers. The primer sequences for the differentiation markers were as follows: Runx2-F, GGACCGACACAGCCATATAAA, Runx2-R, GCCTCATTCCCTAACCTGAAA; BMP2-F, CAGTGGGAGAGCTTTGATGT, BMP2-R, ACCTGGCTTCTCCTCTAAGT; PPARγ-F, GACCTGAAGCTCCAAGAATACC, PPARγ-R, TTCATGTGGCCTGTTGTAGAG; C/EBP-F, CCTCTGGGATGGATCGATTATG, C/EBP-R, GGGACCTTAGTTTCTGGTCTTG. The primer sequences for the TGFβ1/ Smad2 signaling pathway were as follows: Smad2-F, GAGCACGTGAGGTGAGATTT, Smad2-R, CTAAGGACTTCCAGAGGGAAAC; TGFβ1-F, GCAACAATTCCTGGCGTTAC, TGFβ1-R, GTATTCCGTCTCCTTGGTTCAG. TGFβr1-F, CTTCCTTGAGTCACTGGGTATC, and TGFβr1-R, CTTGGCTGTCACCCTAATCTT. The expression levels of target genes were normalized to expression of the GAPDH gene.

MiR-140-5p transient overexpression and inhibition

To assess the effects of miR-140-5p on differentiation, cells were transiently transfected with a vector encoding miR-140-5p mimic or inhibitor (GenePharma, Suzhou, China). Briefly, BMSCs were expanded in culture until 80% confluent, trypsinized, reseeded in 6-well Bioflex plates, washed with PBS, and treated with transfection medium containing riboFECT CP Reagent (RiboBio, Guangzhou, China) and 100 nm of either miR-140-5p mimic, inhibitor, or scrambled miR-NC (control) for approximately 48 h. Total RNA was then extracted for RT-PCR analysis and proteins were extracted for western blot analysis as described.

Luciferase assays of miR-140-5p expression

The putative target sites for miR-140-5p on the 3’-UTR of TGFβR1were predicted using bioinformatics tools. HEK293 cells were seeded on 24-well plates and cultured until 60% confluent. The cells were then transfected with a vector encoding wild type or mutated TGFβR1 3’-UTR using the X-treme GENE™ HP DNA Transfection Reagent. After incubation for 48 h, the cells were lysed in 1× Passive Lysis Buffer and luciferase activities measured using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer´s instructions.

Statistical analysis

All data shown are expressed as mean ± standard deviation (SD) of at least three independent experiments. Group means were compared by independent sample t-tests using Prism version 6 (GraphPad software). A $p \leq 0.05$ (two-tailed) was considered statistically significant for all tests.

Results

Effects of mechanical stimulation on BMSC differentiation

Bone marrow stem cells were induced towards osteogenic or adipogenic differentiation using specific culture conditions with or without additional mechanical stimulation (stretch stress). After 5 days in culture, RT-qPCR and western blotting (WB) were performed to measure expression levels of the BMSC osteogenic markers Runx2 and BMP2 and the adipogenic markers PPARγ and CEBPα. As demonstrated in Figure 1A, cells cultured under osteogenic conditions with mechanical stimulation (stretch stress) exhibited 1.22-fold higher Runx2 mRNA expression and 1.27-fold higher BMP2 mRNA expression than the corresponding no stress group as measured by RT-qPCR (Figure 1Aa-b). Consistent with gene expression levels, WB revealed greater Runx2 and BMP2 protein expression levels in the stretch stress group compared to the no stress group (Figure 1Ac-d). Moreover, cells cultured under adipogenic conditions with stretch stress showed significantly reduced expression levels of the adipogenic marker genes PPARγ and CEBPα than the corresponding no stress group (70% and 72%, respectively, of no stress group values, Figure 1Ba-b), while WB demonstrated parallel reductions in protein expression (Figure 1Bc-d). These results suggest that appropriate stretch stress can promote osteogenic differentiation and inhibit adipogenic differentiation of BMSCs.

Expression of miR-140-5p during osteogenic and adipogenic differentiation of BMSCs

To explore the possible molecular mechanisms underlying the effects of mechanical stimulation on BMSC differentiation, we first assessed the expression levels of miR-140-5p. As shown in Figure 2a and b, miR-140-5p expression gradually decreased and reached a nadir on the third day of osteogenic differentiation. Alternatively, miR-140-5p expression increased significantly during adipogenic differentiation, reaching a peak on the third day, followed by a slight decrease. On day 3, the expression level of miR-140-5p was 20% of control in the osteogenic condition and 5.6-fold higher than control in the adipogenic condition. Thus, miR-140-5p expression app-ears to suppress osteogenic and (or) promote adipogenic differentiation.

We therefore examined if mechanical stress influences miR-140-5p expression. As shown in Figure 2c and d, BMSCs cultured under osteogenic conditions plus stretch stress (A1) demonstrated 24% lower miR-140-5p expression than BMSCs under osteogenic culture without stretch stress (B1), and miR-140-5p expression levels in both osteogenic BMSC groups (A1 and B1) were significantly lower than in BMSCs without induction (A3 and B3). In BMSCs cultured under adipogenic conditions, stretch stress (A2) reduced miR-140-5p expression (by 39%) compared to adipogenic BMSCs without stress (B2). In addition, stretch stress reduced miR-140-5p expression under non-induction culture conditions.

Collectively, these results indicate that the expression level of miR-140-5P is downregulated during osteogenic differentiation of BMSCs and further inhibited by appropriate stretch stress.

Effects of miR-140-5p overexpression on osteogenic and adipogenic differentiation of BMSCs

To provide further evidence for miR-140-5p function in BMSC osteogenic and adipogenic differentiation, we examined the effects of culture condition and stretch stress in BMSCs transfected with a miR-140-5p mimic. RT-PCR on day 3 of induction confirmed miR-140-5p overexpression (Figure 3Aa). Moreover, expression levels of the osteogenic marker genes Runx2 and BMP2 were significantly lower in BMSCs overexpressing miR-140-5p than osteogenic BMSCs transfected with control vector (Figure 3Ac-d), suggesting that miR-140-5p negatively regulates osteogenic differentiation of BMSCs. Further, under adipogenic differentiation conditions, expression levels of the adipogenic marker genes PPAR and C/EBPα were significantly upregulated in BMSCs overexpressing miR-140-5p (Figure 3Ba-b). After 7 days of differentiation under osteogenic conditions, BMSCs overexpressing miR-140-5p (A1 group) also exhibited lower ALP staining intensity than osteogenic BMSCs expressing control vector (A2 group) (Fig. 3C). Conversely, under adipogenic culture conditions, BMSCs overexpressing miR-140-5p (C1 group) demonstrated more intense oil red O staining than adipogenic BMSCs expressing control vector (C2 group). These results suggest that miR-140-5p promotes adipogenic differentiation of BMSCs rather than osteogenic differentiation.

Effects of miR-140-5p knockdown on osteogenic and adipogenic differentiation of BMSCs

We then examined the effect of miR-140-5p knockdown on BMSC differentiation by transfection of a miR-140-5p inhibitor. RT-PCR confirmed knockdown of miR-140-5p compared to control vector (Figure 4Aa). Under osteogenic culture conditions, BMSCs expressing the miR-140-5p inhibitor exhibited significantly higher expression levels of Runx2 and BMP2 than BMSCs expressing control vector (Figure 4Ac-d), while under adipogenic conditions, BMSCs expressing the miR-140-5p inhibitor demonstrated significantly downregulated PPARγ and C/EBPα expression compared to controls (Figure 4Ba-b). Under osteogenic conditions, BMSCs expressing the miR-140-5p inhibitor (A1 group) also exhibited greater ALP staining than cells expressing control vector (A2 group), while under adipogenic conditions, BMSCs with miR-140-5p knockdown (C1 group) exhibited lighter oil red O staining than BMSCs expressing control vector (C2 group). These results provide further evidence that miR-140-5p suppresses osteogenic differentiation of BMSCs and promotes adipogenic differentiation

The participation of TGFβ1/Smad2 signaling pathway

Furthermore, we try to find out the pathway signal targeted to the mechanism that miR-140-5p regulate to BMSCs differentiation. With the help of biomedical date source website, we decided to choose TGF-1/Smad2 as our target signal pathway to perform further research. Firstly, luciferase reporter assay was performed to detect the direct binding between miR-140-5P and TGFβr1. We found that ectopic expression of miR-140-5P significantly decreased the luciferase signal of 3’ UTR of WT TGFβr1 compared to the miR-NC. This suppressive effect was abolished by mutated miR-194 binding site of 3’ UTR TGFβr1. Secondly, The WB expression of TGF-1/Smad2 signal transduction pathway markers significantly downregulate in the BMSCs with adipogenic as significantly upregulated with osteogenic (Figure 5). These results indicated the participating role of TGFβ1/Smad2 signaling pathway in the regulatory role of miR-140-5P on the BMSCs differentiation.

The expression level of TGF-1/Smad2 signal transduction pathway markers in BMSCs change as miR-140-5p overexpression and inhibition under mechanical stimulation

To explore the connection between miR-140-5P and TGF-1/Smad2 signal pathway, we transfected the miR-140-5P mimic and inhibitor into BMSCs separately and found TGF-1/Smad2 pathway were influenced by the change activity of miR-140-5P. As mentioned above, during the osteogenic differentiation of BMSCs, the activity of TGFβ1/Smad2 marks was significantly promoted under stress. As shown in Figure 6A, this effect was further promoted when agonizing miR-140-5p, which was antagonized in inhibiting miR-140-5p. Conversely, it was observed in adipogenic differentiation that stress inhibited the activity of TGFβ1/Smad2 channels, while the effect is antagonized by a 140-5p inhibitor (Figure 6B). This suggests that the regulation of the TGF-1/Smad2 pathway by miR-140-5p is involved in the regulation of stress on the differentiation of BMSCs.

Discussion

Osteoporosis is a systemic bone disease characterized by reduced bone mineral density and bone mass [21]. During the development of osteoporosis, the proportion of BMSCs differentiated into solitary cells is reduced and bone marrow is gradually replaced by adipose tissue [22]. Previous studies have shown that adipocytes and osteoblasts share a common progenitor cell [23]. Under the appropriate mechanical (stretch) stimulation, a variety of extracellular matrix components are secreted in the stretch zone and BMSCs differentiate into osteoblasts [24]. Consistent with previous research results, we demonstrate that appropriate mechanical stimulation can promote osteogenic differentiation of BMSCs and inhibit the adipogenic differentiation of BMSCs[13, 14]. Adipogenic differentiation was associated with enhanced expression of miR-140-5p and concomitant downregulation of TGFβ1/Smad2 signaling components (TGFβ1, TGFβR1, and Smad2), while both mechanical stimulation and osteogenic culture suppressed miR-140-5p expression and upregulated expression of TGFβ1/Smad2 signaling components.

Stress exists in all intracellular environments, regulating cell biological functions, such as cell proliferation and differentiation, and appropriate stress stimulation is particularly important in bone regeneration after fracture[25]. To further study the effects of mechanical stimulation on the differentiation of bone marrow stem cells in vitro, we developed a sinusoidal tensile stress loading machine suitable for stretch stimulation of cultured BMSCs[20]. Our results clearly show that appropriate mechanical stimulation can promote osteogenic differentiation of BMSCs as indicated by deeper ALP staining and upregulation of osteogenic markers Runx2 and BMP2. Runx2 is a member of the Runxx family of transcription factors and acts as a specific transcription factor for bone cells during development and remodeling [26], while BMP-2 is a member of the TGFβ structure-related protein superfamily that can induce bone and cartilage formation in combination with bone conduction carriers such as collagen and synthetic hydroxyapatite [27]. Our results also demonstrated that the same extent of mechanic stimuli inhibited the adipogenic differentiation of BMSCs, indicated by the lower red oil O staining, as well as upregulated expressions of osteogenic gene markers including PPAR-γ and C/EBPα, which are the most important transcription factors in MSCs during adipogenic differentiation. Our results

were consistent with previous study by Turner et al., which showed that the appearance of intracellular lipid droplets was delayed in the stress group during the differentiation and maturation of MSCs into adipocytes [28], and supported the statement that shear stresses at physiological levels can differentiate MSCs into endothelial cells by inducing the expression of endothelial-cell-specific markers [29].

The early phase of BMSC differentiation involves changes in signaling pathways that in turn alter responses to external factors. MicroRNAs are small non-coding RNA of about 20 to 24 nt [30] with diverse functions in stem cells including regulation of development, differentiation, proliferation, and apoptosis. MicroRNA-503-5p has been shown to inhibit osteogenic differentiation of stem cells under stress [31]. Wang et al. used gene chips to detect changes in miRNAs during stem cell differentiation, and found that the expression level of miR-140 increased significantly during adipogenic differentiation [32]. Similarly, Zhang and colleagues demonstrated that the expression level of miR-140-5p was three times higher during the third stage of adipogenic differentiation of adipose-derived stem cells compared to control cells [33], suggesting the varied regulatory roles of the same miRNA in the differentiation of stem cells. To elucidate the underlying mechanisms, we examined the expression of miR-140-5p at different stages of BMSC differentiation, the effects of miR-140-5p overexpression and knockdown on differentiation, and the associations among miR-14-5p expression, osteogenic and adipogenic marker expression, and TGFβ1/Smad2 signaling component expression under osteogenic and adipogenic culture conditions in the presence and absence of mechanical stress. Under osteogenic culture conditions, mechanical stimulation inhibited the expression of miRNA-140-5p, upregulated TGFβ1/Smad2 signaling components, and promoted osteogenic differentiation of BMSCs, proving a possible explanation for the benefits of appropriate mechanical stress on bone remodeling.

In accord with our results, Liu at al. reported that reduced expression of miR-503-5p promoted the osteogenic differentiation of bone marrow stromal cells under stress [31]. Other miRNAs that regulate stem cell differentiation and are influenced by mechanical stress include miR-34 s, miR-29b, and miR-30a [34–36]. Various miRNAs can have unique regulatory effects on osteogenic differentiation and respond differently to mechanical stress. Overexpression and knockdown of miR-140-5p in BMSCs resulted, respectively, in enhanced osteogenic and adipogenic differentiation. In addition, TGFβR1 expression was reduced by miR-140-5p overexpression compared to negative control cells under both culture conditions in the presence and absence of mechanical stress; in contrast, TGFβR1 expression was enhanced by miR-140-5p knockdown under all conditions. Furthermore, luciferase reporter assay confirmed direct binding between miR-140-5p and the TGFβR1 promoter. Intriguingly, TGFβR1 expression was elevated by mechanical stress compared to the corresponding unstressed group regardless of miR-140-5p expression level, indicating that both stress and miR-140-5p can regulate BMSC differentiation through reciprocal regulation of the TGFβ1/Smad2 signaling pathway.

Taken together, mechanical stimuli promote osteogenic differentiation of BMSCs, in combing with lower expression of miR-140-5p via TGFβ1/Smad2 signaling pathway, suggesting the positive role of mechanical stimuli in the differentiation of BMSCs towards osteoblasts.

Conclusions

Appropriate mechanical stimulation promotes the osteogenic differentiation of BMSCs by lowering the expression of miR-140-5P, which in turn upregulates the TGFβ1/Smad2 signaling pathway and downstream osteogenic genes. Our results provide a plausible explanation for the beneficial effects of mechanical stimulation on bone remodeling and a foundation for the development of more effective strategies to treat osteoporosis.

Abbreviations

BMSCs: bone marrow mesenchymal stem cells

ALP: alkaline phosphatase

BMP2: bone morphogenetic protein-2

C/EBPα: Recombinant Human CCAAT/enhancer binding protein

PPARγ: peroxisome proliferators-activated receptor-γ

RUNX2: Runt-related transcription factor 2

Smad2: drosophila mothers against decapentaplegic2

TGF-β: transforming growth factor-β

Declarations

Ethics statement

Ethics approval and consent to participate: All animal experiments were conducted according to relevant national and international guidelines and approved by the Animal Care and Use Committee of Southern Medical University. Appropriate steps were taken to ameliorate suffering.

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Conflicts of interest:

None

Consent for publication:

None

Authors' contributions:

YP Q and GZ Z, ZL Y carried out the experiment. YP Q and GZ Z. wrote the manuscript with support from RG L and CJ Z. RG L and CJ Z. supervised the project. All authors have read and approved the manuscript.

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Figures

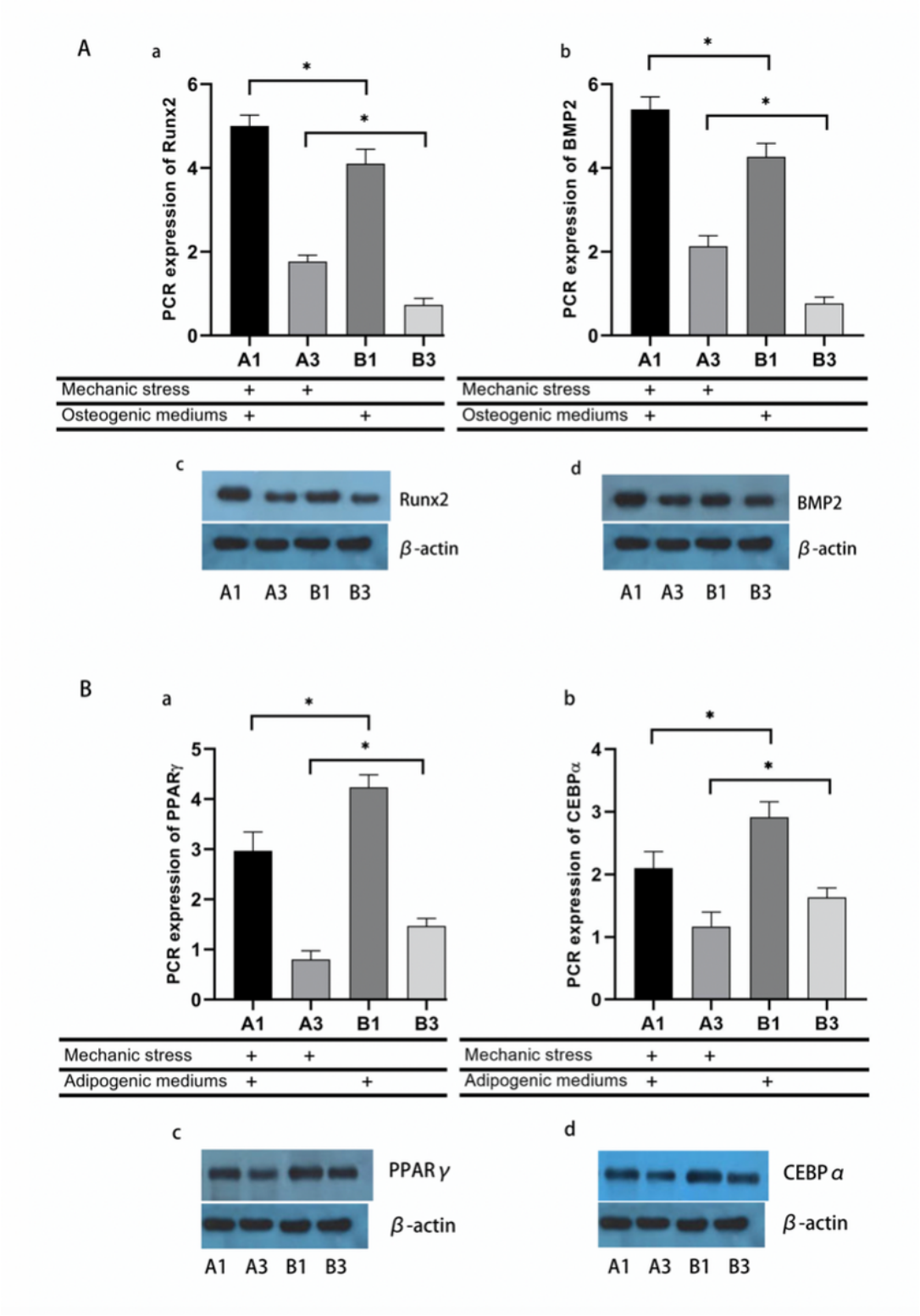

Figure 1

Mechanical stimulation (stretch stress) promotes osteogenic differentiation and suppresses adipogenic differentiation of bone marrow stem cells (BMSCs). A) Expression levels of the bone osteoblast markers Runx2 and BMP2 under osteogenic culture conditions in the presence (+) and absence of stretch stress. B) Expression levels of the bone adipogenic markers PPARγ and CEBPα under adipogenic culture conditions in the presence and absence of stretch stress.

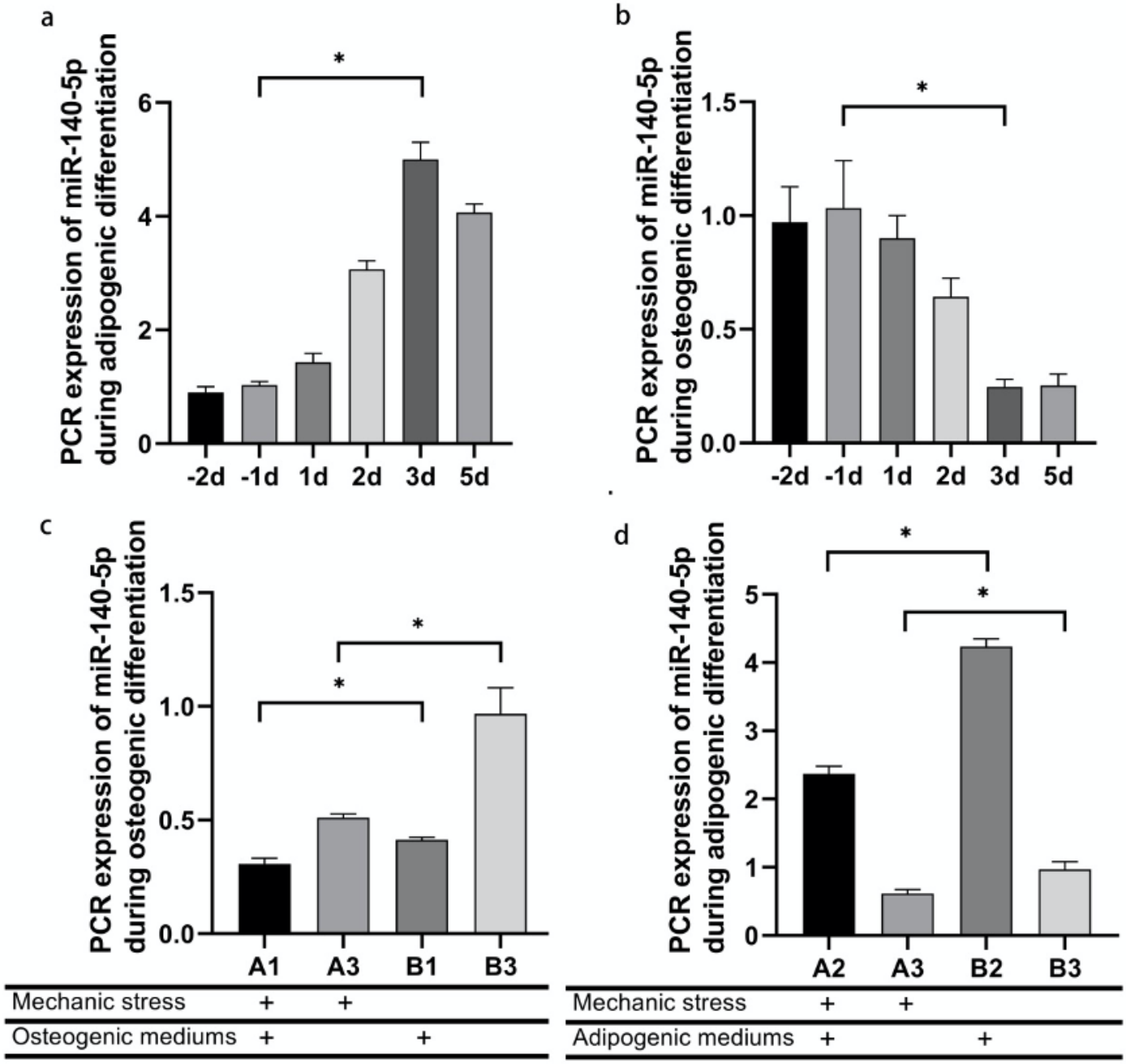

Figure 2

Expression of miR-140-5p in BMSCs is increased by adipogenic induction medium and mechanical stress (stretch stress) and reduced by osteogenic differentiation medium and stretch stress. A) Expression of miR-140-5p during osteogenic differentiation of BMSCs. B) Expression of miR-140-5p during adipogenic differentiation of BMSCs. C) Effects of mechanical stress on miR-140-5p expression in BMSCs during osteogenic differentiation and under non-induction culture conditions. D) Effects of mechanical stress on

miR-140-5p expression in BMSCs during adipogenic differentiation and under non-induction culture conditions.

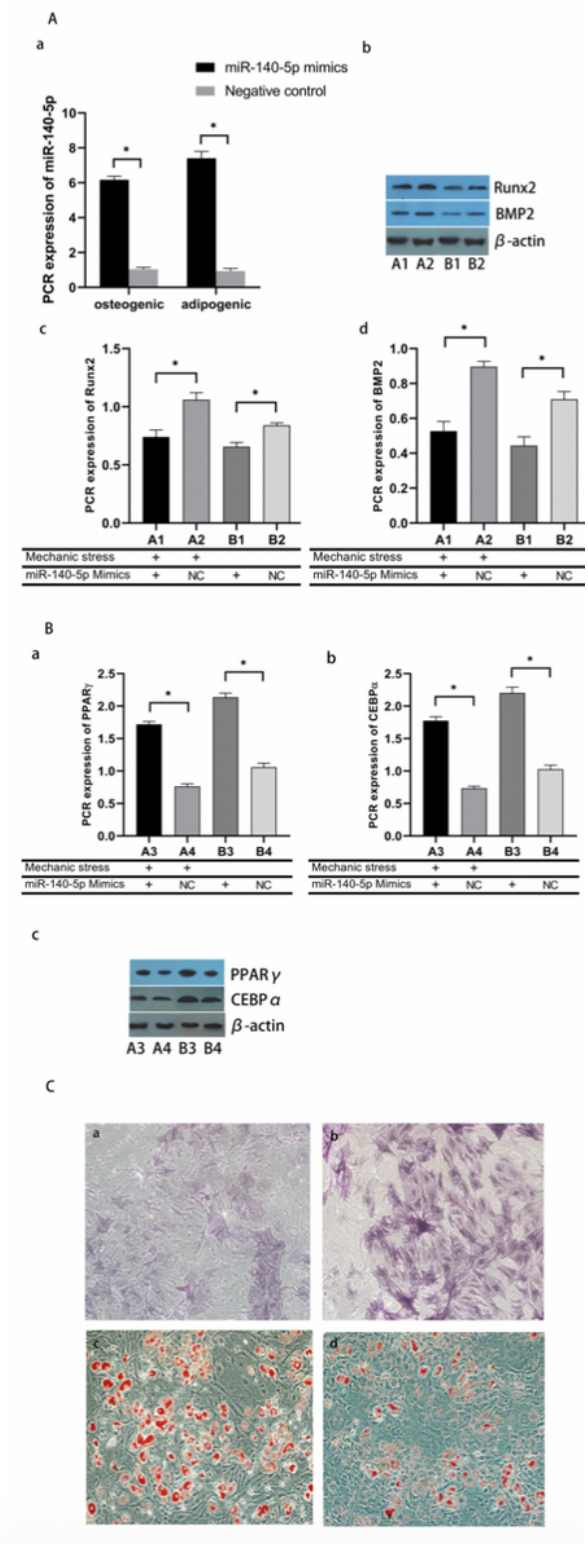

Figure 3

Overexpression of miR-140-5p promotes adipogenic and suppresses osteogenic differentiation of BMSCs. A-a) RT-PCR validation of miR-140-5p mimic overexpression on day 3 of osteogenic and adipogenic culture. A-bcd) RT-PCR and western blot (WB) analyses of osteogenic Runx2 and BMP2

expression in BMSCs overexpressing miR-140-5p mimic (day 3). B-abc) Expression of adipogenic PPARγ and C/EBPα in BMSCs overexpressing miR-140-5p mimic (day 3). C) Alkaline phosphatase (ALP) staining of BMSCs on day 7 of osteogenic culture and oil red O staining on day 10 of adipogenic culture.

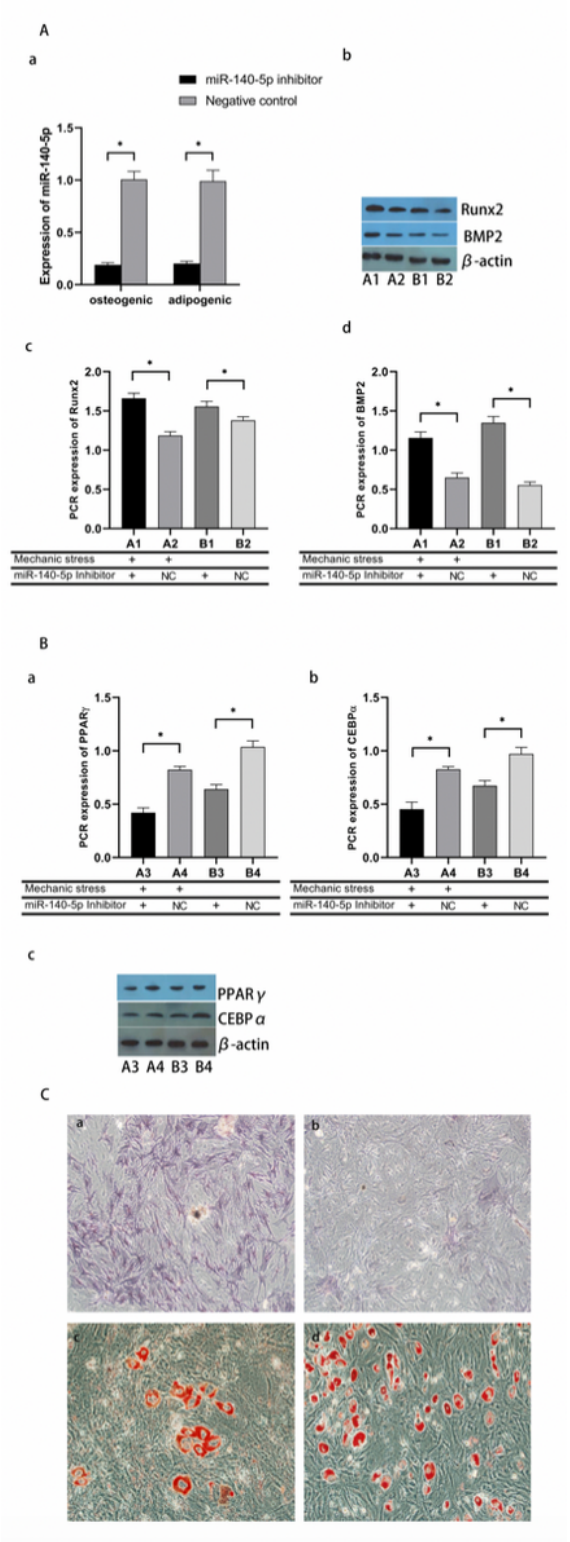

Figure 4

A miR-140-5p inhibitor promotes osteogenic and suppresses adipogenic differentiation of BMSCs. A-a) RT-PCR validation of miR-140-5p knockdown on day 3 under both osteogenic and adipogenic culture

conditions. A-bcd) RT-PCR and WB analyses of osteogenic Runx2 and BMP2 expression levels in miR-140-5P knockdown BMSCs on day 3 compared to cells expressing negative control vector. B-abc) Expression of adipogenic PPARγ and C/EBPα in miR-140-5p knockdown BMSCs on day 3. C) ALP staining of BMSCs on day 7 and oil red O staining on day 10.

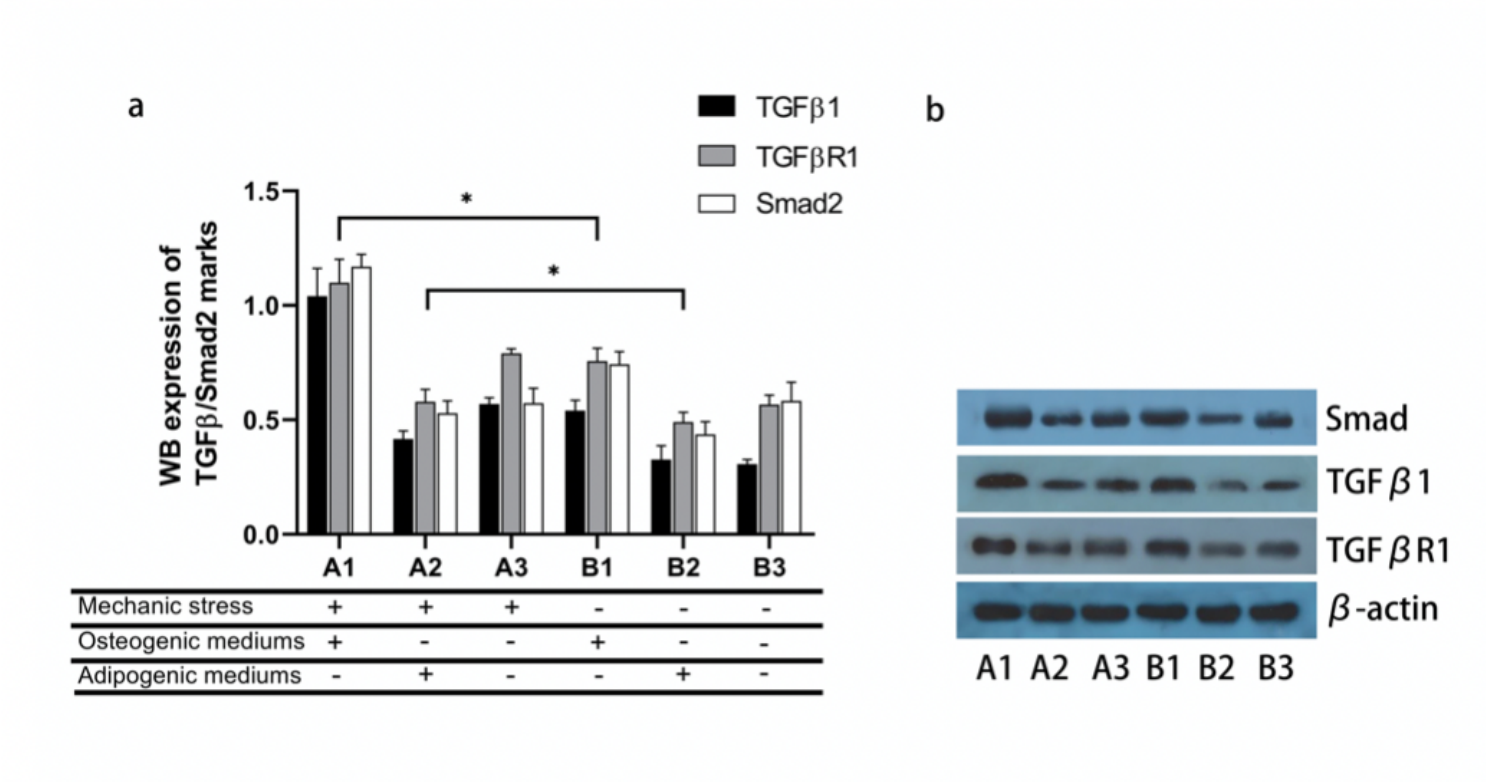

Figure 5

Stretch stress enhances expression levels of TGFB1/Smad2 signaling pathway components TFG1, TFGR1, and Smad2 in BMSCs on day 3. a) WB results for protein expression. b) Sample western blot.

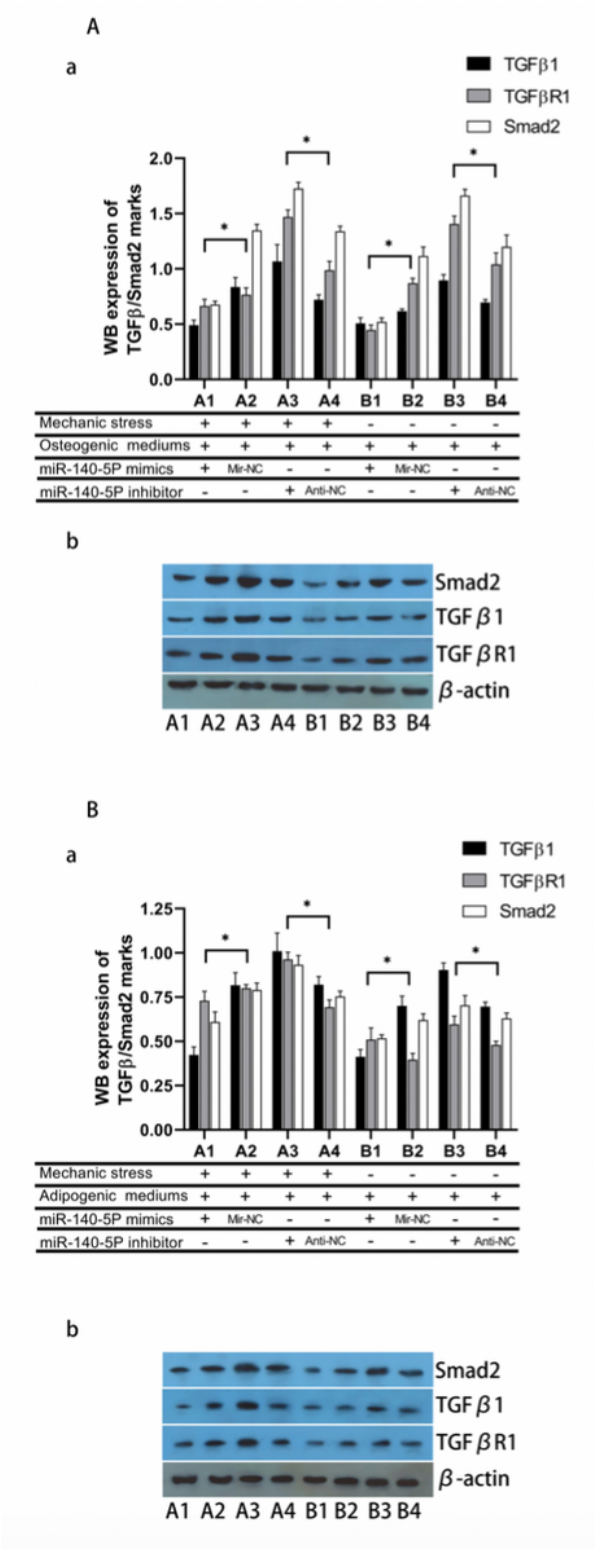

Figure 6

Stretch stress and miR-140-5p knockdown enhance expression of TGF-1/Smad2 signaling pathway components under osteogenic and adipogenic differentiation conditions. Western blot (WB) results of TGF-1/Smad2 pathway component expression in BMSCs with miR-140-5p overexpression/ inhibition under osteogenic (A) and adipogenic differentiation conditions (B).

Supplementary Files

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